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THE GROWTH OF THE GONOCOCCUS IN VARIOUS GASEOUS ENVIRONMENTS

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That micro-organisms are sensitive to respiratory gases has been discussed for a number of years. Pasteur showed that, in the free access of oxygen, the yeast plant fermented the sugars very slowly; while if grown in the presence of a large excess of CO_2, fermentation proceeded at its maximum pace. This alone shows the effect of respiratory gases on fermentation.

C. Fränkel[1] showed that some bacteria, namely, B. typhosus and Friedländer's bacillus, grow as well under pure CO_2 as in atmospheric conditions; other strains, such as B. prodigiosus, B. proteus and B. phosphorescens will grow under pure CO_2 but not as luxuriantly as under aerobic conditions; while still a third group will not grow at all under pure CO_2 namely, the spirillum of Asiatic cholera, B. anthracis, Micrococcus tetragenus, the bacteria of chicken cholera and of rabbit and mouse septicemia. Of this last group, all will grow in a mixture of CO_2 and air, or some will grow under pure CO_2, if the temperature of incubation is raised. He also says that the same holds true in a general way in atmospheres of pure oxygen.

Ferran[2] and Belfonti[3] found that the tetanus bacillus could be successfully habituated to aerobic conditions by the gradual increase of oxygen in cultures. We know that as a rule most anaerobes are sensitive to exposure to oxygen, yet here is a case in which an anaerobic bacterium could be transformed into an aerobic one.

Wherry and Oliver[4] claimed that the gonococcus when first isolated grows best under a partial tension produced by the hay bacillus.

Wherry and Ervin[5] showed that the removal of CO_2 inhibited the growth of a recently isolated tubercle bacillus.

Cohen and Markle,[6] Cohen,[7] and Cohen and Fleming[8] published several articles on the advantage of using a reduced oxygen tension and an increased CO_2 tension in the isolation of the meningococcus.

Chapin[9] and Kohman[10] also reported on the advantage of CO_2 in the growth of the gonococcus and the meningococcus, but St. John[11] claimed that the

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[1] Ztschr. f. Hyg. u. Infectionskr., 1889, 5, p. 332.

[2] Centralbl. f. Bakteriol., 1898, 24, p. 28.

[3] Arch. per le. sc. med., 16, cited by Hiss and Zinsser.

[4] J. Infect. Dis., 1916, 19, p. 288.

[5] Ibid., 1918, 22, p. 194.

[6] Jour. Am. Med. Assn., 1916, 67, p. 1302.

[7] Ibid., 1918, 70, p. 1999.

[8] Jour. Infect. Dis., 1918, 23, p. 337.

[9] Ibid., 1918, 23, p. 342.

[10] Jour. Bacteriol., 1919, 4, p. 571.

[11] Med. Rec., 1919, 95, p. 184.

respiratory gases were not an important factor in the growth of the meningococcus; stating, however, that the advantage of the method as suggested by Wherry and Oliver was due to increase in moisture from sealing the tube.

Gates[12] claimed that the advantage of Cohen's method for the isolation of the meningococcus was due to the increased moisture content obtained by sealing the tube. Gates and Kohman thought that the effect of CO_2 on the growth was not due to direct influence on respiration but to the action of the CO_2 on the medium tending to acidify alkaline medium, thus providing a delicate means of regulating the H-ion concentration.

Lately Swartz and Davis[13] described what they believe to be a simpler method of producing partial tension (by heating the tube) than the method involving the use of the hay bacillus. Further, it seems from their work that partial tension is a more satisfactory method for isolation of the gonococcus than the aerobic method.

In view of the conflicting ideas held by various workers as to the relative value of the influence of the gaseous environment, moisture and reaction on the primary cultivation of the gonococcus, we decided to try to analyze the effect of these factors.

Technic.—In these experiments two strains were used. Strain A, from a case of gonorrheal ophthalmia, was isolated on ascites agar under partial tension with the hay bacillus (fig. 1). Strain B was isolated in the same way from a case of vulvovaginitis.

The medium used with culture A was NaCl ascites agar with a P_H 7.4; and with culture B, Na_2HPO_4 ascites agar with a P_H 7±. The agar was made with beef infusion broth containing 1% of peptone (Difco) and 0.5% salt or sodium phosphate. The ascites fluid was added in the proportion of one part to four of the tubed and sterilized agar.

The gases used were obtained in steel cylinders. A mixing chamber, as shown in fig. 2, was devised for procuring the proper mixture of the gases. The culture tubes used were of large size, fitted with two hole rubber stoppers, through which a short and a long glass tube were passed as shown in fig. 3. The free ends of the glass tubes were fitted with gum rubber tubing and screw clamps. All connections were sealed with a mixture of paraffin and Canada balsam (see bottom of illustrations).

1. *Experiments.*—The stock cultures grown since the time of isolation under partial tension (hay bacillus) were transplanted to various gaseous environments to determine whether growth would occur under such sudden environmental changes. The results were:

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[12] Jour. Exper. Med., 1919, 29, p. 321.

[13] Jour. Am. Med. Assn., 1920, 75, p. 1124.

It will be noted that when derived from a partial tension culture, the gonococcus grew best under similar conditions or under pure hydrogen, and that growth did not occur under high concentrations of CO_2 or O_2; however, as will be shown later, it can be adapted to do so.

TABLE 1

RESULT OF TRANSPLANTATION OF STOCK CULTURES

Series A Source, From Partial Tension		Series B Source, From Partial Tension		
Gas Environment	Growth in 24 Hours	Gas Environment	Growth in 24 Hours	Growth in 48 Hours
100% H_2	+	Aerobic	+	+
100% CO_2	—	Partial tension	+++	+++
50% H_2, 50% CO_2	—	Anaerobic (Wright's)	—	—
75% H_2, 25% CO_2	±	100% H_2	+	++
100% O_2	—	CO_2 100%	—	—
50% O_2, 50% N_2	—	O_2 100%	—	—
50% O_2, 50% H_2	—			

— No growth
± Just perceptible growth
+ Delicate growth
++ Fair growth
+++ Good growth
++++ Very luxuriant growth

2. *Adaptation to Hydrogen and the Tenacity of this Adaptation.*—1. It was noted that the gonococcus would grow readily under H_2 directly from partial tension (table 1). This obviated the necessity of slowly adapting the organism to this gas. Transplants were made daily and maintained under hydrogen for periods varying from 1½ to 3 months before the following experiments were made (table 2).

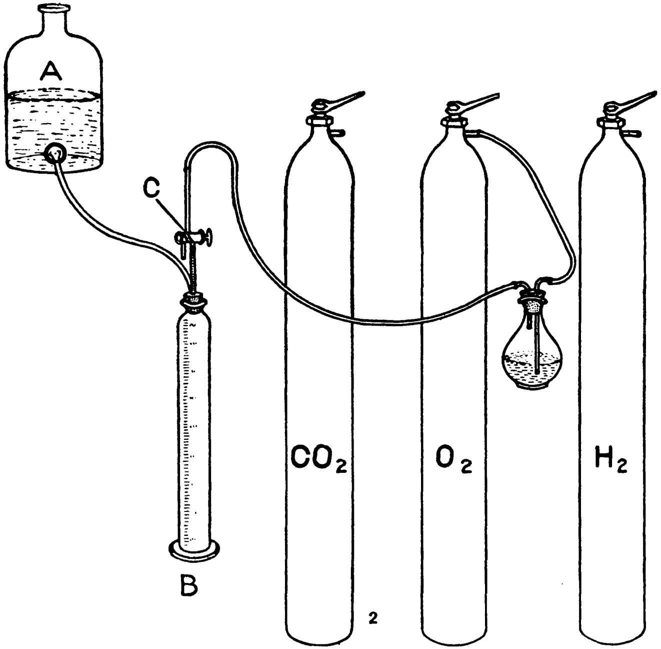
TABLE 2

EXPERIMENTS WITH TRANSPLANTS FROM H_2

Series A				Series B			
Gas Mixtures	Hours			Gas Mixtures	Hours		
	24	48	72		24	48	72
Air (sealed tube)	—	+	++	Air (not sealed)	—	±	++
100% CO_2	—	—	—	100% CO_2	—	—	—
60% air, 20% H_2, 20% CO_2	—	±	++	100% O_2	—	—	—
Anaerobic (Wright's)	—	—	—	Anaerobic (Wright's)	—	—	—
100% H_2	+++	+++	+++	Anaerobic (Wright's) dextrose medium	—	—	—
				Partial tension	++	++	++

It will be noted that such a strain adapted to grow under H_2 had its growth inhibited by the presence of O_2 or CO_2 and grew best under H_2 or the partial tension method. When O_2 was removed from the air by pyrogallic acid and caustic soda no growth occurred.

3. *Adaptation to Oxygen.*—In the following successive subcultures the amount of O_2 in the air was increased; each transplant representing a subculture from the preceding one.



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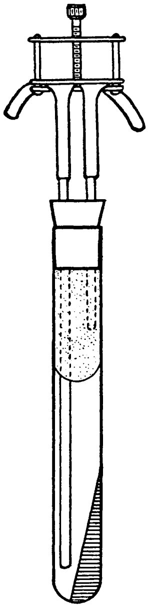

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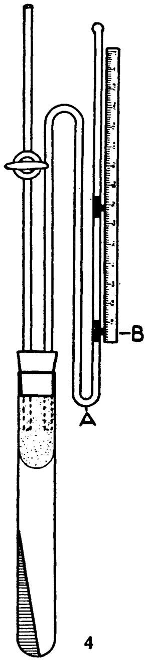

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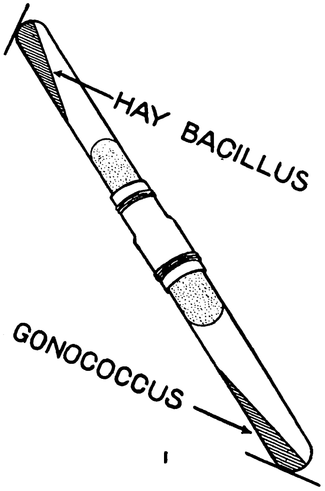

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Fig. 1.—Apparatus used to isolate strain A from a case of gonorrheal ophthalmia isolated on ascites agar under partial tension with hay bacillus.

Fig. 2.—The apparatus used in producing the various gas mixtures. The gases used were obtained in steel cylinders as illustrated. From these cylinders they were passed first to the wash bottle, which contained water, and merely indicated the rate of flow of the gases. From there the tube led to the graduated cylinder B, through a two way stopcock C, which permitted all air to be driven from the tubes before the gases were admitted to the mixing chamber (B), which was filled with water. Gases were admitted to B through a short glass tube and were measured by displacement of water to the reservoir A through a long glass tube reaching from the bottom of the cylinder B to the rubber tube connecting the reservoir.

The water level in the reservoir was 4 feet above the base of the mixing chamber when no gasses were in the cylinder, and only 2 inches higher when all the water in the cylinder had been displaced. Therefore the variations in the pressure on the gas volumes amounted only to approximately the height of the cylinder B. This was corrected in the graduation of the cylinder. The water in the system was made acid to prevent absorption of CO_2 when that gas was used.

After the desired percentages of gases had been introduced into the chamber and mixed, the tube from the tanks was withdrawn and a short, curved glass tube was attached to the top of the cylinder B, the inlet now becoming the outlet.

Fig. 3.—The culture tube. The long arm was thoroughly flamed and slipped past the cotton plug. The short arm did not penetrate the plug which was packed tightly around the long arm. To the short arm was attached the tube from the mixing chamber and the gas mixture under pressure from the reservoir was allowed to displace the air in the culture tube. Regardless of the specific gravity of the gas, it was always introduced through the short arm, being thus filtered through the cotton plug. The plane of the culture tube was altered depending on the specific gravity of gas mixture. The air was allowed to escape through the long arm. If gasses were introduced through the long arm, and so unfiltered through the cotton, contamination invariably resulted. Many times the amount of gas necessary to fill the tube was passed through, to avoid as much as possible the error of allowing air to remain in the cotton plug or elsewhere, altering the gas concentrations.

Fig. 4.—Apparatus used in Exper. 6.—All connections were sealed with Canada balsam and paraffin. The tube with the glass stopcock was attached to a vacuum pump and air removed until the manometer A recorded several mm. less than atmospheric pressure. The stopcock was closed and the tube incubated in this condition.

In series A the transplants were made from the earliest appearing colonies. In series B the subcultures were made from colonies appearing in 24-48 hours. Transplant 8 (Series B) was carried to O_2 without air several times. The growth was in all instances delayed, and subcultures failed after the first or second transplants.

Summarizing table 3, we may conclude that the gonococcus may be adapted to growth in concentrations of O_2 above those which previously inhibited its growth.

TABLE 3

RESULTS OF TRANSPLANTATION

Series A First Transplant from Aerobic Culture				Series B First Transplant from Partial Tension		
Gas Mixtures	Hours			Gas Mixtures	Hours	
	24	48	60		24	48
1. 20% O_2, 80% air	++	..	..	1. 25% O_2, 75% air	++	+++
2. 50% O_2, 50% air	—	±	++	2. 25% O_2, 75% air	++	+++
3. 50% O_2, 50% air	—	±	++	3. 35% O_2, 65% air	++	++
4. 50% O_2, 50% air	±	+	++	4. 50% O_2, 50% air	+	++
5. 50% O_2, 50% air	+	+	..	5. 65% O_2, 35% air	+	++
6. 65% O_2, 35% air	±	+	++	6. 75% O_2, 25% air	+	++
7. 100% O_2	±	+	++	7. 80% O_2, 20% air	±	++
8. 100% O_2	±	+	++	8. 86% O_2, 14% air	±	++
9. 100% O_2	±	+	++	9. 100% O_2	±	+
				10. 100% O_2	—	—

TABLE 4

TENACITY OF ADAPTATION TO OXYGEN

Series A All Transplants from 100% Oxygen				Series B All Transplants from 80% O_2, 20% Air			
Gas Mixtures	Hours			Gas Mixtures	Hours		
	24	48	72		24	48	72
Air	++	++	..	100% CO_2	—	—	..
100% H_2	—	—	—	Air	+	++	..
100% CO_2	—	—	—	100% H_2	—	±	bubbles in medium
75% H_2, 25% CO_2	—	—	—				

Having determined that such an adaptation to high concentration to O_2 could be brought about, the following experiments were made in order to study the respiratory requirements of such an adapted strain (table 4).

It is evident from comparison of table 4 with the preliminary experiment (table 1) that an O_2 adapted strain grows more readily under aerobic conditions than an unadapted culture, especially one from partial tension. Further experiments showed that such an aerobic subculture could be transplanted back to the high O_2 concentration providing that the culture in the first instance had been kept under O_2 for a sufficient length of time.

The action toward other gaseous environments is unchanged except that it seems to grow less under hydrogen.

4. *Adaptation to CO_2.*—As shown in table 2, the gonococcus grown aerobically cannot be transferred suddenly to a high concentration of CO_2 ($4\,CO_2 + 1$ air) but the experiments in table 5 show that such an adaptation can be gradually brought about. One strain was maintained in 48-hour subcultures in almost pure CO_2 for two months. The transplants are derived each from the preceding one:

TABLE 5

ADAPTATION OF TRANSPLANTS TO CO_2

Series A First Transplant from Aerobic Culture			Series B First Transplant from Partial Tension			
Transplant	Gas Mixture	24 Hours	Transplant	Gas Mixture	24 Hours	48 Hours
1	CO_2 14%, air 86%	++	1	CO_2 25%, air 75%	+	++
2	CO_2 18%, air 82%	++	2	CO_2 40%, air 60%	+	++
3	CO_2 30%, air 70%	++	3	CO_2 50%, air 50%	+	+
4a	CO_2 86%, air 14%	—	4	CO_2 50%, air 50%	++	..
4b	CO_2 30%, air 70%	++	5	CO_2 60%, air 40%	++	..
5	CO_2 37%, air 63%	++	6	CO_2 66%, air 34%	++	..
6	CO_2 37%, air 63%	++	7	CO_2 75%, air 25%	++	..
7	CO_2 43%, air 57%	++	8	CO_2 80%, air 20%	++	..
8	CO_2 50%, air 50%	++	9	CO_2 86%, air 14%	++	..
9	CO_2 50%, air 50%	++	10	CO_2 100%	++	..
10	CO_2 66%, air 34%	++	11	CO_2 100%	±	+
11	CO_2 75%, air 25%	++	12	CO_2 100%	++	..
12a	CO_2 100%	—				
12b	CO_2 80%, air 20%	—				
12c	CO_2 75%, air 25%	++				

Tenacity of Adaptation to CO_2.—When adapted to high concentrations of CO_2 the strain is unfavorably affected by O_2 but still retains its ability to grow under partial tension and H_2. In a few instances in which growth occurred under aerobic conditions, subcultures were not viable (table 6).

TABLE 6

EQUALITY OF ADAPTATION TO HIGH CONCENTRATIONS OF CO_2

Series A All Transplants from CO_2/Air = 3/1				Series B All Transplants from CO_2 ("pure")		
Gas Mixtures	Hours			Gas Mixtures	Hours	
	24	48	60		24	48
100% H_2	—	±	+	100% H_2	—	+
20% air, 80% H_2	±	+	++	100% O_2	—	—
Aerobic	—	—	—	Partial tension	++	++
Air (sealed tube)	—	±	++	Air (open tube)	—	—

5. *Factors Concerned in Delayed Growth.*—It will be noted that in the experiments made to test the tenacity of the adaptation of the carbon dioxid and hydrogen strains, in certain cases there was delayed growth of the subcultures (tables 2 and 5). The following set of experiments was devised to study the factors producing this delayed growth (table 7).

TABLE 7

EXPERIMENTS MADE TO SHOW FACTORS PRODUCING DELAYED GROWTH

Series A All Transplants from CO_2/Air = 3/1			Series A All Transplants from 100% H_2			
Gas Mixtures	Result in Hours		Gas Mixtures	Result in Hours		
	24	48		24	48	72
50% CO_2, 50% O_2..............	—	+	CO_2 50%, O_2 50%..........	—	±	..
66% CO_2, 34% O_2..............	±	+	H_2 50%, CO_2 50%..........	±	±	..
50% CO_2, 50% H_2	+	+	H_2 66%, CO_2 34%..........	+	+	..
50% O_2, 50% H_2...............	—	—	H_2 25%, O_2 75%...........	—	—	+
			H_2 50%, O_2 50%...........	—	±	..
			H_2 66%, O_2 34%...........	—	+	..
			H_2 75%, O_2 25%...........	—	++	..

From study of table 7 on strains adapted to CO_2 and H_2, it appears that oxygen seems to be an important factor in producing delayed growth.

6. *Study of the Enclosed Gases.*—The next question presenting itself concerned quantitative changes in the enclosed gases due to respiration of the organisms. In order to determine whether gas is produced by the organisms, they were transplanted into a closed tube connected with a mercury manometer shown in fig. 4. A pressure of several mm. less than the atmospheric was produced by a vacuum pump and the cultures incubated. The results are shown in table 8.

TABLE 8

RESULTS OF TRANSPLANTATION OF ORGANISMS INTO CLOSED TUBE CONNECTED WITH MERCURY MANOMETER

Series A Transplant from H_2 Environment for 3 Months			Series B Transplant from Partial Tension		
Time in Hours	Reading 37 Degrees	Growth	Time in Hours	Reading 37 Degrees	Growth
0	83 Mm.	—	0	90 Mm.	—
14	66 Mm.	—	24	60 Mm.	—
24	41 Mm.	±	36	33 Mm.	±
30	25 Mm.	+	48	0 Mm.	++
42	0 Mm.	++			

The same apparatus connected to an uninoculated tube of sterile medium was incubated as a control for 3 days, and no changes in pressure occurred. From table 8 it seems that bacteria respire before growth appears and growth is not visible until considerable change in the gaseous environment has occurred.

An attempt was made to determine the nature of these gaseous changes. An analysis of the gas from a 48-hour aerobic subculture grown under air, sealed with a rubber stopper and fitted with double glass stopcocks was made, with the following results: Culture CO_2, 6%, O_2 2.5%; control (laboratory air) CO_2 0.5%, O_2 19%. Residual gas; noncombustible in either case.

An analysis of gas from a 30-hour growth under hydrogen, transplanted from a H_2 adapted strain showed: Culture: CO_2 3%, O_2 0.2%; control (tube filled with H_2) CO_2 0%, O_2 0.6%. Remainder combustible in both cases.

It seems that O_2 certainly is used by the gonococcus and CO_2 given off in aerobic and H_2 cultures.

7. *The Effect of Moisture Compared with that of Gases.*—Method: Four transplants were made: aerobic; to its adapted gas requirement; under its gas requirement with $CaCl_2$ in the neck of the tube; aerobic in desiccator containing water, the desiccator being opened to air frequently. The purpose of this series of transplants was to put the organism under its previous gas requirement with and without moisture; and ignoring the gas requirement—with moisture and without moisture (table 9).

TABLE 9

RESULTS OF EXPERIMENTS ON FOUR TRANSPLANTS CONCERNING EFFECT OF MOISTURE WITH EFFECT OF GASES

Previous Gas History	Gaseous Environment, Etc.	Result in Hours	
		24	48
Series A:			
1 Adapted to 75% CO_2	Aerobic	—	..
2 Adapted to 75% CO_2	75% CO_2	+++	..
3 Adapted to 75% CO_2	75% CO_2 + $CaCl_2$	++	..
4 Adapted to 75% CO_2	Aerobic + water in desiccator	—	..
1 Adapted to 98% H_2	Aerobic	—	..
2 Adapted to 98% H_2	98% H_2	+++	..
3 Adapted to 98% H_2	98% H_2 + $CaCl_2$	++	..
4 Adapted to 98% H_2	Aerobic + water in desiccator	±	..
Series B:			
1 Partial tension	Aerobic	+	+
2 Partial tension	Partial tension	+++	+++
3 Partial tension	Partial tension + $CaCl_2$	++	++
4 Partial tension	Aerobic + water in desiccator	+	+

A repetition of these experiments produced similar results. Although it is plain that moisture plays some rôle, it is also evident that the gases play an important part.

8. *Study of Viability under Gases.—(a)* Nov. 30, 1919, a culture of the gonococcus was transplanted to ascites agar and incubated under H_2 until Dec. 14, 1919, at which time a subculture from it grew, but on Dec. 22 a transplant from the latter growth showed no viability.

(b) Following this experiment repeated tests were made to determine viability under hydrogen. A typical instance is reproduced in the following data. The strain used for this experiment had been transplanted frequently under H_2 for a month. Of 5 transplants on ascites agar, incubated under H_2 for 48 hours, then allowed to remain at room temperature for 3 days, one was subcultured daily for viability with the following results (table 10).

TABLE 10

RESULTS OF SUBCULTURING TRANSPLANT FOR VIABILITY

Subculture From	Growth in Hours		Viability
	24	48	
Tube 1	++	++	5 days
Tube 2	++	++	6 days
Tube 3	+	++	7 days
Tubs 4	±	+	8 days
Tube 5	±	±	9 days

These results would indicate that viability under hydrogen is dependable up to one week. Dr. J. Hermanies (personal communication), in maintaining a large number of gonococcus strains, has shown that viability is increased under partial tension as opposed to aerobic conditions. The increased viability under these conditions may be due to decrease in oxygen.

SUMMARY AND CONCLUSIONS

Cultures of the gonococcus isolated at partial tension by the aid of the hay bacillus grew under pure H_2 but not under CO_2 or O_2. When grown for some weeks under pure H_2 they seemed to become more sensitive to the deleterious influence of O_2 and CO_2, but still grew well by the partial tension method (hay bacillus). Although growth occurred under almost pure H_2, it failed when anaerobic conditions were produced by means of caustic soda and pyrogallic acid.

While strains isolated at partial tension will not grow under pure O_2 or pure CO_2, they can be adapted to do so.

It was noted during these adaptation experiments that the strains were able to adapt themselves to a new gaseous environment more rapidly than they lost old adaptations; e. g., although a strain could be adapted to grow under CO_2, it required a prolonged series of subcultures under CO_2 to make it lose its ability to grow in the presence of O_2. A study of the delayed growth appearing in certain gas mixtures showed that an excessive supply of O_2 probably played an important part in producing the inhibition. This is supported by an analysis of the enclosed gases in a sealed culture, showing that growth begins after a certain amount of O_2 is consumed and CO_2 given off.

While moisture must play an important part, the experiments show that according to the previous gaseous adaptation of the micro-organisms their growth will be favorably or unfavorably influenced by various gas mixtures.

All cultures, regardless of their adaptation, would grow under partial tension as furnished by the hay bacillus and those adapted to H_2 or CO_2 would grow better at partial tension than under aerobic conditions.

In the continuous cultivation of the gonococcus under the various gases the viability was greatest under H_2 and least under O_2 or aerobic conditions.

As shown by Wherry and Oliver, Wherry and Ervin, Cohen and Markle and others, certain strains of the gonococcus and meningococcus grow best at partial tension when first isolated from the body; and these experiments on adaptation emphasize the importance of including the partial tension method of cultivation in the routine isolation of parasitic bacteria.

We believe that the well recognized fact that bacteria encountered in Nature are aerobic, partial tension, anaerobic, or facultative in their respiratory requirements, is an expression of their previous gaseous adaptation.